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CONTROL OF SALIVARY GLAND DEGENERATION IN THE IXODID TICK *Amblyomma hebraeum*

by

ROBERT ARTHUR HARRIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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Abstract

Salivary gland function was studied in the tick *Amblyomma hebraeum* Koch (1844) (Acari : Ixodidae) using a simple physiological assay. Isolated salivary glands, which had their main salivary ducts ligated, were incubated for 15 minutes in Tissue Culture Medium 199' containing 10μM dopamine. Wet weights were taken before and after the incubation, with the increase in weight taken as the index of secretory function. This assay is sensitive enough to detect induced salivary gland degeneration both in salivary glands of small (0.20-0.30g) partially fed ticks implanted into replete ticks, and salivary glands from small (0.20-0.30g) partially fed ticks which had been joined in parabiosis to replete ticks.

Salivary glands from both small partially fed ticks and replete ticks lost secretory function within 4 days post-removal; however, unlike replete ticks, small ticks neither totally lost salivary function, nor did they exhibit the characteristic ultrastructural changes associated with salivary gland degeneration.

In vivo salivary gland degeneration also occurred in large (>0.40g) partially fed ticks, and was blocked either by severing all the opisthosomal nerves, or by removing the

---

'The medium was modified as follows: NaCl was added (2.10g/l)to adjust the medium to 360mOs; also, the medium was bufferred to pH 7.3 using 10mM MOPS buffer (Sigma)

seminal receptacle. However, subsequent replacement of the seminal receptacle, after extirpation, resulted in normal salivary gland degeneration. Thus, there is a neural component in the pathway controlling salivary gland degeneration which is located in the opisthosomal nerves. A chemical factor which is released from the seminal receptacle also influences salivary gland degeneration. Normal salivary gland autolysis was induced in large partially fed ticks which had their seminal receptacles removed, by injecting a crude extract of genital tracts taken from fed males. However, injecting extracts of male salivary glands into such ticks did not have this effect. This indicated that a specific chemical factor, found in the male genital tract, was transferred during mating, resulting in salivary gland degeneration.

Salivary glands from large partially fed ticks put into organ culture up to 12 hours after removal from the host did not lose secretory function (tested 96 hours after removal from the host). However, salivary glands from such ticks, when put into culture 24 hours or more after removal from the host, underwent complete degeneration. Thus, it would appear that once salivary gland degeneration has been triggered, it is irreversible.

A dose dependent loss of salivary gland function occurred in ticks kept in organ culture in the presence of a mixture of ecdysone and 20-hydroxyecdysone. Salivary gland function was lost both in ticks cultured with only ecdysone

or 20-hydroxyecdysone.

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Table of Contents

Chapter — Page

I. Chapter One
General introduction 1

Statement of the Problem 3

II. Chapter Two

Development of the Assay for Secretory Competence 5

A. Introduction 5

B. Materials and Methods 8

1) Animals 8

a) Larvae 8

b) Nymphs 8

c) Adults 8

2) Feeding 9

3) Assay For Secretory Competence of Salivary Glands 9

4) Organ Transplant 10

5) Parabiosis of ticks 11

6) Electron Microscopy 12

7) Statistics 13

C. Results 14

D. Discussion 17

III. Chapter Three

Neural Involvement in the Control of Salivary Gland Degeneration 31

A. Introduction 31

B. Materials and Methods 33

1) Procedure for Staining Neural Tissue 33

2) Surgical Procedure for Nerve Disruption .33

C. Results 35

D. Discussion 38

IV. Chapter Four
Involvement of Mating in the Control of Salivary Gland Degeneration 45

A. Introduction 45

B. Materials and Methods 47

1) Surgery 47

2) Injection of Male Tissue Extracts 47

C. Results 49

D. Discussion 51

V. Chapter Five 55

A. Introduction 55

B. Materials and Methods 57

1) Organ culture 57

C. Results 59

D. Discussion 62

VI. Chapter Six 70

A. Biological Significance 73

B. Suggested Experiments 75

VII. Literature Cited 81

VIII. Appendix 90

List of Figures

Figure 1 - Apparatus used to retain the gut diverticula within the haemocoel during surgery.....20

Figure 2 - Increase in weight over the first 25 minutes for three isolated salivary glands.....22

Figure 3 - Loss of secretory ability as a function of time off the host for replete ticks and small partially fed ticks.....24

Figure 4 - Secretory ability of salivary glands of small partially fed ticks implanted into replete ticks and into small partially fed ticks.....26

Figure 5 - Secretory ability of salivary glands of small partially fed ticks joined in parabiosis to replete ticks or small partially fed ticks.....28

Figure 6 - Ultrastructural appearance of the secretory labyrinth of a small partially fed *Amblyomma hebraeum* which had been off the host for 5 days.....30

Figure 7 - Structure of the synganglion of *Amblyomma hebraeum*.....41

Figure 8 - Effect of severing groups of nerves on salivary gland degeneration in large partially fed ticks 44

Figure 9 - Effect of removal of the seminal receptacle on salivary gland degeneration, as well as the effect of injection of extracts of male tissues on salivary gland degeneration in large partially fed ticks 54

Figure 10 - Secretory ability, after 4 days, of salivary glands of large partially fed ticks put into organ culture at various times after removal from the host..67

Figure 11 - Secretory ability of salivary glands from

large partially fed ticks cultured with various concentrations of ecdysteroids.....69

Figure 12 - *In vitro* preparation used in previous studies to monitor salivary fluid secretion.....78

Figure 13 - Ultrastructural appearance of the secretory labyrinth of a replete *Dermacentor variabilis*.....80

List of Tables

Table 1 - Groups of nerves which were cut in the nerve disruption experiment.....42

Table 2 - Secretory ability of salivary glands from ticks kept in organ culture for 2 and 4 days, as well as the effects of culturing large partially fed ticks with their gut diverticula intact.....65

Table 3 - Equation used to calculate ^{36}Cl transport in isolated salivary glands.....92

I. Chapter One

General introduction

Blood sucking arthropods are faced with certain unique problems associated with feeding. First, such ectoparasites face reprisals from the host which attempts to prevent feeding. Most blood sucking animals counteract this by feeding for a matter of seconds or minutes. After departing the host, excess water is excreted in comparative safety. Familiar hematophagous insects as the dipteran *Aedes aegypti* and the hemipteran *Rhodnius prolixus* achieve fed to unfed weight ratios of approximately 2:1 and 6:1, respectively (Gwadz, 1969; Goodchild, 1955), whereas ixodid ticks may increase their body weight more than 100 fold (Balashov, 1972). It was perhaps necessary, in order to accomplish this remarkable feat, that ixodid ticks have resorted to a protracted feeding pattern which includes bouts of diuresis interspersed throughout the feeding period. To minimize the threat of host reprisals over the 7 to 10 day feeding period, the ticks actually cement their mouthparts into the integument of the host, in order to prevent being dislodged by any but the most dedicated attempts.

Diuresis in *Amblyomma hebraeum* is not performed by the Malpighian tubules as in most other haematophagous arthropods; rather, it is accomplished by the salivary glands periodically during feeding (Gregson, 1967; Tatchell,

1967; Kaufman & Phillips, 1973a). The salivary glands secrete the excess imbibed fluid back into the host.

The salivary glands are composed of 3 types of acini. The type I acini are believed to be involved in uptake of water vapour from the atmosphere, when the unfed tick is in negative water balance (Rudolph & Knülle, 1974; McMullen *et al*, 1976). Formation of the attachment cement and the secretion of salivary enzymes and anticoagulants have been ascribed to the type II and type III acini (Chinery, 1973; Binnington, 1978). The type III acini are believed to perform the osmoregulatory function during feeding (Meredith & Kaufman, 1973; Binnington, 1980; Fawcett *et al*, 1981b).

Feeding can be divided into two phases (Snow, 1969), a slow phase lasting 6 to 7 days and a rapid phase lasting 18 to 20 hours. At the onset of feeding, the salivary glands are incapable of secreting the large volumes of saliva required for osmoregulation (Kaufman, 1976). However, during the slow phase of feeding a complex cellular re-organization occurs in the type III acini, which is necessary for fluid secretion to occur (Fawcett *et al*, 1981a). During this period a number of other developmental changes also occur. For example, endocuticle is deposited (Lees, 1952), Gene's organ and the ovaries develop (Till, 1961), and the nerves grow to accommodate subsequent stretch (unpublished observations). During the slow phase of feeding, female *Amblyomma hebraeum* increase in body weight from approximately 0.03g (unfed) to about 0.30-0.40g. Development

of the fluid secretory ability is complete before the tick reaches a body weight of 0.20g (Dr. W.R. Kaufman, Department of Zoology, University of Alberta; personal communication).

Upon termination of feeding the salivary glands are resorbed within several days (Till, 1961). Resorption is a result of intense autophagic activity in the type III acini (Harris & Kaufman, 1981), primarily in the ablumenal interstitial cell and the f-cell (terminology after Fawcett *et al*, 1981a; also see fig. 12).

Degeneration of the salivary gland is triggered by a haemolymph-borne factor: 'tick salivary gland degeneration factor' (TSGDF; Harris & Kaufman, 1981). However, the identity and origin of this factor are unknown. In addition, nothing is known about the mechanisms controlling the release of TSGDF. Harris & Kaufman (1981) found that female *Amblyomma hebraeum* under 0.30g were not subject to salivary gland degeneration, while females over 0.40g did undergo salivary gland degeneration. This suggested that some cue associated with feeding influences the release of TSGDF. Also, salivary glands from unmated females over 0.40g do not degenerate, indicating that mating also influences the release of TSGDF (Kaufman, 1983).

Statement of the Problem

In light of the above, I wished to answer two major questions associated with salivary gland degeneration. I

first attempted to determine what some of the physiological signals triggering the release of TSGDF were; and second, I was able to test a potential candidate for TSGDF.

Chapter 2 of this thesis is concerned with the development of a physiological method to assay salivary gland function. Experiments dealing with the determination of the neural components involved with the release of TSGDF are discussed in Chapter 3. The influence of mating on salivary gland degeneration is dealt with in Chapter 4, while in Chapter 5 I will suggest a candidate for TSGDF.

II. Chapter Two

Development of the Assay for Secretory Competence

A. Introduction

Harris and Kaufman (1981) demonstrated that salivary gland degeneration was controlled hormonally. They induced degeneration in salivary glands of partially fed female *Amblyomma hebraeum* 0.20-0.30g (hereafter referred to as small partially fed ticks) by implanting the glands of small partially fed ticks into the haemocoels of fully engorged ticks and by joining small partially fed ticks to engorged ticks in parabiosis. Salivary gland degeneration was determined by the presence of autophagic vacuoles in the area of the type III acinus which is believed to be responsible for fluid secretion: namely the labyrinth formed by the interdigitations of the f-cell and the ablumenal interstitial cell.

In the same study, salivary gland degeneration was also monitored by measuring changes in the levels of acid phosphatase and succinate dehydrogenase, marker enzymes for lysosomes and mitochondria respectively. These biochemical

assays were able to detect normally occurring salivary gland degeneration, but not degeneration which had been induced experimentally in the salivary glands from small partially fed ticks (Harris, unpublished observations). Thus, I decided that a physiological assay based on fluid transport might prove more promising. Kaufman (1976) showed in *Dermacentor andersoni* that the rate of fluid secretion of isolated salivary glands stimulated by dopamine declined significantly after termination of feeding. Active chloride transport was postulated to be the driving force behind salivary gland fluid secretion (Kaufman and Phillips, 1973c; Sauer *et al*, 1974; Saur *et al*, 1976). I initially attempted to measure dopamine-stimulated ^{36}Cl transport. As outlined in the appendix, this assay proved unsuccessful. However, while incubating these isolated salivary glands I noticed that many increased in size, presumably due to the transport of fluid into the lumen. This suggested the second assay, by which one might monitor salivary gland function by simply measuring net weight increase in dopamine stimulated salivary glands which had their main salivary ducts ligated.

The size of the blood-meal appears to have an effect on salivary gland degeneration. Harris & Kaufman (1981) noted that salivary glands of small partially fed ticks do not undergo the ultrastructural changes associated with degeneration for at least two days, nor do they exhibit the characteristic biochemical changes of degeneration for 5 days after removal from the host. However, salivary glands

from engorged ticks demonstrate marked ultrastructural[1] and biochemical changes as early as 2 days off the host. I attempted to confirm this difference between engorged ticks and small partially fed ticks physiologically. As well, I tested whether the physiological assay would be able to detect salivary gland degeneration which had been experimentally induced.

---

[1]The characteristic ultrastructural changes are often apparent in ticks immediately upon detachment from the host, indicating that salivary gland degeneration may be initiated before the end of feeding. A significant loss of fluid transporting ability is not apparent, however, until 1 day post detachment.

B. Materials and Methods

1) Animals

Ticks used in this study were obtained from a colony established with specimens originating from the Veterinary Research Institute, Onderstepoort, Republic of South Africa. The colony was maintained in darkness at 26°C, 95% relative humidity.

a) Larvae

Larvae were fed at least 3 weeks after the eggs had hatched. The fed larvae were returned to incubation and allowed to moult into nymphs, which normally took 3 weeks.

b) Nymphs

Nymphs were stored for a minimum of 3 weeks after moulting. Feeding usually required 7-8 days. Fed nymphs were then returned to storage and left to moult into adults, which usually occurred within 4 weeks.

c) Adults

Newly moulted adults were transferred to clean vials and stored for at least 4 weeks before being fed.

2) Feeding

For feeding, ticks were confined to rabbits as described by Kaufman and Phillips (1973a). Briefly, a foam rubber corral topped with fine woven cloth was cemented with latex (Latex Compounding Co., Toronto, Canada) to a shaven area on the backs of mature rabbits. Since rabbits develop an immune resistance after a single exposure to feeding *Amblyomma hebraeum* (Bowessidjaou *et al*, 1977), only naive rabbits were used.

Adult females of *Amblyomma hebraeum* would not readily attach without the presence of feeding males; therefore, both males and females (approximately 30 of each) were confined on each rabbit. Males were allowed to attach and feed for several days before the females were introduced.

3) Assay For Secretory Competence of Salivary Glands

Ticks were immobilized by gluing them with cyano-acrylate glue (Aron Alpha, Tagosi Chemical Co., Japan) to the bottom of a small petri dish lined with adhesive tape. The tick was flooded with tissue culture medium (TCM 199; Gibco) modified as follows: the sodium bicarbonate was omitted, and the medium was buffered (pH 7.3) with 10mM morpholinopropanesulfonic acid (MOPS; Sigma) and adjusted to 360 mOsm with 2.1g NaCl/l.

The dorsum was removed with the aid of a razor blade scalpel, and the main salivary ducts were located, ligated with 8-0 silk (Davis & Geck), and severed distal to the ligature. The glands were then removed to fresh medium for approximately 15 minutes. Wet weights (the glands weighed approximately 2-8mg) were taken on a Sartorius 2474 micro balance 60 seconds after the glands had been blotted according to a standard procedure. Each gland was then transferred to a container containing TCM 199 with 10μM dopamine, a concentration which stimulates the maximal rate of fluid secretion (Kaufman, 1976). During incubation the medium was agitated by slow stirring with a magnetic stirrer. After incubation, the net increase in weight was taken as an index of secretory competence.

4) Organ Transplant

Donor ticks were immobilized as already described, flooded with TCM 199, and the dorsum removed. The main salivary ducts were located, freed of the surrounding tissue and severed at their junction with the pharnyx. The glands were stored in fresh medium while recipient ticks were prepared. All recipient ticks were anaesthetized on ice for 10 minutes. A 5μl disposable glass pipette was sealed with paraffin wax and slipped inside a 100 μl glass pipette, thus forming a microsyringe. The gland to be implanted was drawn

up in a small volume of medium with the microsyringe. The mouth of the syringe was then inserted into a small incision made in the dorsum of the recipient tick, and sealed into place with glue. After the glue had set, the gland was injected into the haemocoel of the recipient tick. The 5 µl pipette was then sealed to the 100 µl pipette with a small amount of glue. Prepared ticks were stored in darkness, at 26°C and 95% relative humidity. After 2 days, the implanted glands were dissected out and placed in fresh medium. The main salivary duct was then located and ligated with 8-0 silk. The resident glands of the recipient ticks were likewise prepared, and all glands were tested for secretory competence.

5) Parabiosis of ticks

Partially fed ticks were chilled on ice for 10 minutes, after which a circular piece of cuticle approximately 15-20 mm^2 was removed. A corresponding opening was made in the integument of engorged females restrained in a hypobaric chamber (see fig. 1), such that the opening was exposed to atmospheric pressure. In this way, air pressure maintained the gut diverticula within the haemocoel. The partially fed ticks were positioned so that the opening in the cuticle was opposite the opening in the dorsum of the engorged tick. The partially fed tick was secured in this position with glue.

Control pairs consisting of two small partially fed ticks were prepared in a similar manner with the exception that both ticks were chilled, making the vacuum apparatus unnecessary. Patency was determined 3 days later by injecting ^{14}C-labelled inulin (a suitable haemolymph space marker; Kaufman et al, 1980) into one member of the pair. Injections were made with an 'Agla' micrometer syringe (Burroughs-Wellcome) fitted with a 30 gauge disposable needle. The needle was inserted through an articulation between the capitulum and the scutum. Three hours later haemolymph samples were taken from both ticks and mixed with 'Scintiverse' scintillation cocktail (Fisher Scientific). Samples were counted in a Beckman LS 9000 Scintillation Counter. After the haemolymph samples were taken, the glands of both ticks were harvested and assayed for secretory competence.

6) Electron Microscopy

Salivary glands were dissected out under TCM 199, and the large tracheae separated. The glands were fixed for 12 hours in 2.5% gluteraldehyde in 0.05M cacodylate buffer (pH 7.2) made up in 2.5% sucrose. After washing again in buffer, the glands were post-fixed in 4% OsO_4-0.05M cacodylate buffer for 1 hour. After washing in buffer, the tissue was stained *en bloc* in aqueous uranyl acetate (2%) for 20

minutes. The tissue was then passed through an ascending series of ethanol concentrations for dehydration, followed by 2 changes of propylene oxide (100%). Tissues were infiltrated overnight in Araldite 502-propylene oxide (1:1, v/v) and washed twice with pure Araldite 502. The tissue was then transferred to plastic moulds, covered with Araldite 502 and polymerized at 60°C for 48 hours. Thin sections were cut with glass knives on a Porter-Blum Ultramicrotome and mounted on copper grids (150 mesh). Grids were stained in 5% uranyl acetate (in methanol) for 25 minutes and counter-stained in lead citrate (Reynolds, 1963) for 2 minutes. The grids were then examined on a Phillips 200 transmission electron microscope.

7) Statistics

All means were compared using a Student's t-test. Significance is indicated at the $p<0.05$ or $p<0.01$ level.

C. Results

Fluid uptake as a function of time was measured for salivary glands of small partially fed ticks within 1 hour of removal of the tick from the host. The increase in weight appeared to be linear for the first 20 minutes of incubation (fig. 2). On the basis of these results, a 15 minute incubation period was adopted in order to ensure a constant rate of fluid uptake.

Salivary glands incubated in dopamine-free medium did not change weight significantly (0.32±0.21mg/gland/15min, n=11), indicating that fluid uptake was not due to simple osmosis.

I next tested whether the assay could distinguish between non-degenerating and degenerating salivary glands. Glands from small partially fed ticks lost secretory function over the first 4 days post-removal to a plateau which was maintained for at least 15 days. This drop, to only 30% of the initial secretory ability, was not, however, as severe as the drop detected in glands from engorged ticks (0.94-3.13g). Virtually all secretory ability of such glands was lost by the third day after detachment (fig. 3). In some experiments, small partially fed ticks were removed from the host for 4 days in order to permit the salivary glands to reach the plateau level of secretion. The ticks were then allowed to re-attach and feed for 2 days. Salivary glands

from these ticks regained much of their lost function (fig. 3).

Using ultrastructural criteria, Harris and Kaufman (1981) demonstrated that autolysis could be induced in glands from partially fed ticks which had been implanted into the haemocoels of engorged ticks. I repeated those experiments in order to determine if the physiological assay described here was able to detect induced degeneration. Figure 4 illustrates the results of these experiments. There was a marked reduction of secretory competence in salivary glands from small partially fed females implanted into engorged ticks, as compared to similar glands implanted into other small partially fed ticks. However, secretory rates of all glands were reduced as compared to unoperated small partially fed ticks. Upon dissecting each tick, I noticed that the implanted gland was compressed into a tight mass. This mechanical trauma may have had an adverse effect on the secretory ability of the implanted glands, as well as of the resident glands which were also compacted to some degree. If this were the case, one would expect a more pronounced effect in the control ticks where space in the haemocoel is at a premium. Indeed, figure 4 shows that secretion by resident glands of implanted control ticks was very depressed compared to that of glands from unoperated small partially fed ticks, whereas resident glands of implanted engorged ticks showed almost no depression at all as compared to their unoperated counterparts.

To alleviate this problem, I attempted to join, in parabiosis, small partially fed ticks with engorged ticks. Parabiosis avoided compaction of the salivary glands, and had the added attraction of maintaining a tracheal supply to both pairs of glands. Secretory competence was tested after 3 days of parabiosis. Figure 5 shows that fluid uptake by glands from small partially fed ticks joined to engorged ticks was significantly reduced compared to glands of small partially fed ticks which came from control pairs. Fluid uptake by control glands was virtually identical to that by salivary glands from unoperated, small, partially fed ticks. Glands from both members of the experimental pairs secreted slightly more than glands from unoperated engorged ticks (fig. 5).

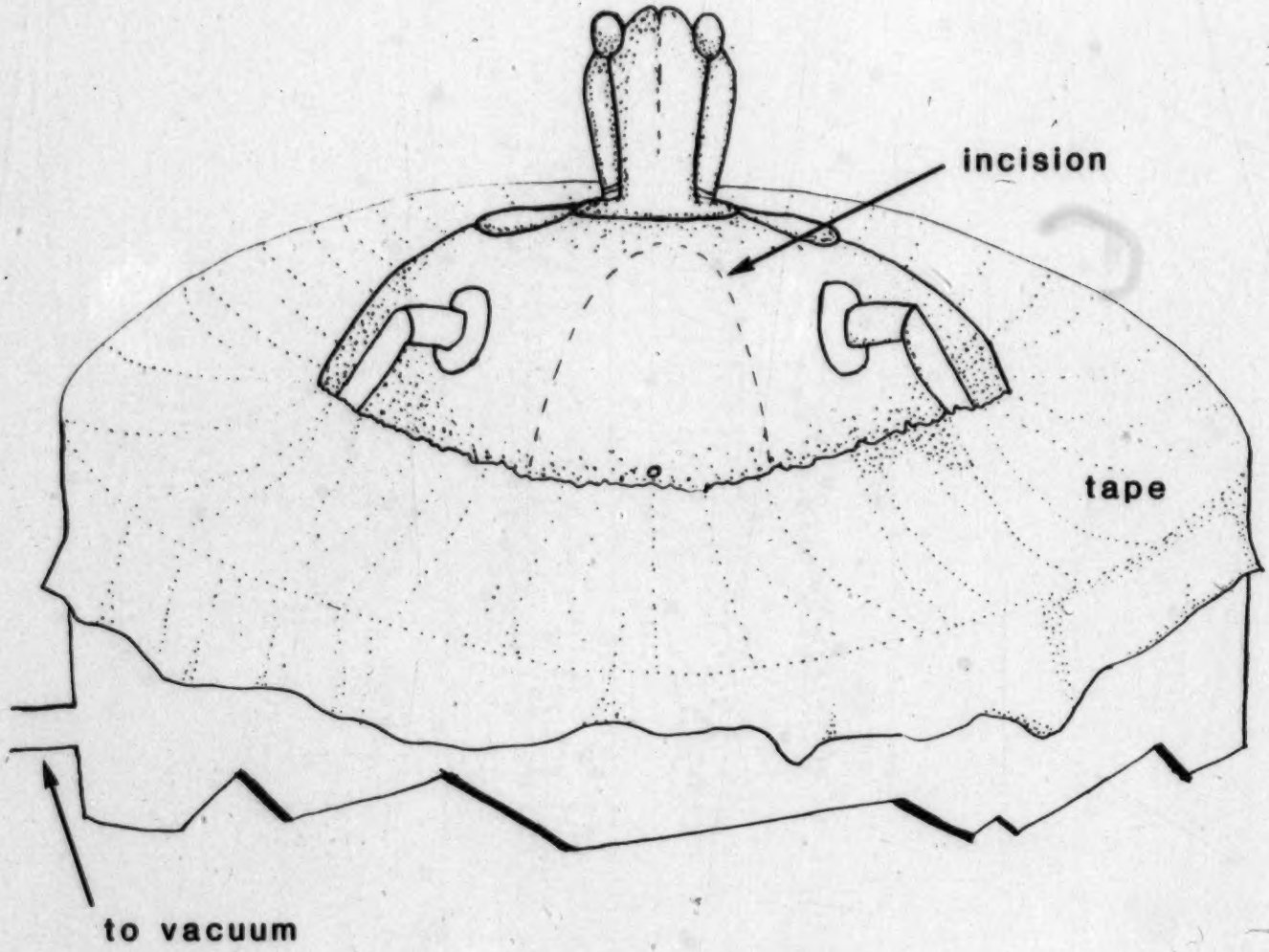
D. Discussion

Previous attempts in this laboratory to develop biochemical assays with which to monitor salivary gland degeneration were not completely successful. Such assays, based on changes in the levels of succinate dehydrogenase (a mitochondrial marker enzyme) and acid phosphatase (a lysosomal marker enzyme), were not sensitive enough to detect degeneration which had been experimentaly induced in tick salivary glands (Harris, unpublished observations). However, the physiological assay described here detected induced degeneration successfully. The minor difficulties encountered when implanting salivary glands in an attempt to induce degeneration probably resulted, not from a fault in the assay, but rather from a problem associated with transplanting a very large organ into a restricted space. The slight, yet statistically insignificant ($p>0.05$), inhibition of degeneration found in glands from both members of an experimental pair parabiosed together may have been a result of a drop in the hormone titer when the haemolymph volume of the partially fed female was combined with the haemolymph of the engorged tick, or more likely it might reflect a temporary inhibition of the release of TSGDF caused by the surgical trauma.

Although salivary glands from small partially fed ticks lost much secretory ability when removed from the host, this

loss does not appear to be caused by the same process which caused degeneration in salivary glands from engorged ticks, for at least two reasons. First, the characteristic cytological changes which accompany the loss of function in salivary glands of engorged ticks do not manifest themselves in salivary glands of small partially fed ticks for at least 5 days (fig. 6). Yet, it was during this 5 day period that most of the activity was lost in glands from such ticks. Secondly, the autophagic disruption which is associated with degeneration would appear to be irreversible, whereas the loss of secretory ability in glands from small partially fed ticks was reversible to a great degree (fig. 3). The loss of secretory competence in small partially fed ticks may be due to a loss of catalytic adenylate cyclase activity as well as a loss of dopamine sensitive adenylate cyclase activity, both of which drop to a plateau level in salivary glands over the first 4 days after removal (W.R. Kaufman, personal communication).

Fig. 1. Illustration of apparatus used to retain gut diverticula within the haemocoel during surgical procedures. The incision indicated allowed access to both the synganglion and the seminal receptacle. For parabiosis, the tick was oriented such that the dorsal surface of the idiosoma was exposed through the tape. The vacuum was adjusted so as to just maintain the gut diverticula within the tick. After completion of surgery, the incision was sealed with glue. (Diagram courtesty of Ms. Sarah Barnes, Office of Community Relations, University of Alberta)


incision
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to vacuum

Fig. 2. Fluid uptake by 3 isolated salivary glands, taken from small partially fed ticks immediately after removal from the host, incubated in TCM 199 with 10μM dopamine. Note that fluid uptake appears to be linear for at least the first 20 minutes.

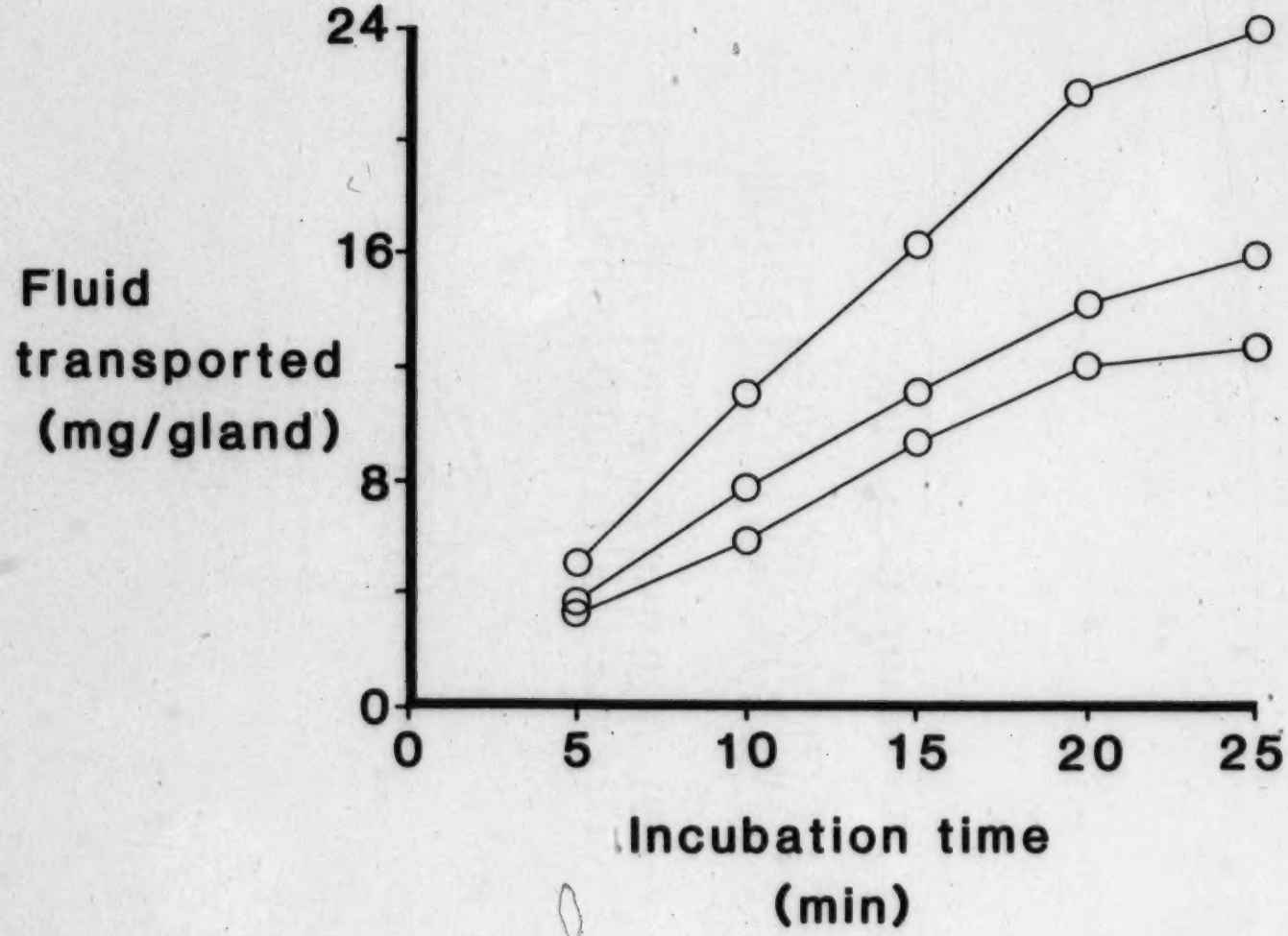

24
16
8
0
Fluid
transported
(mg/gland)
0
5
10
15
20
25
Incubation time
(min)

Fig. 3. Reduction of secretory competence of salivary glands as a function of time post-removal of tick from the host. ( ) partially fed ticks, 0.20-0.30g. ( ) engorged ticks, 0.94-3.13g. ( ) partially fed ticks 0.20-0.30g removed from the host for 4 days and allowed to re-attach and feed for 2 days. Mean, SE and N are indicated for each point. Statistical differences are indicated as follows: (*) $0.01>p>0.001$, (**) $p<0.001$ (Student's t-test). In glands from partially fed ticks, secretory competence reaches a plateau level approximately 30% of that from glands of ticks freshly removed from the host, and remains there for at least 10 more days. Partially fed ticks allowed to resume feeding 4 days after removal from the host, substantially recovered lost secretory competence.

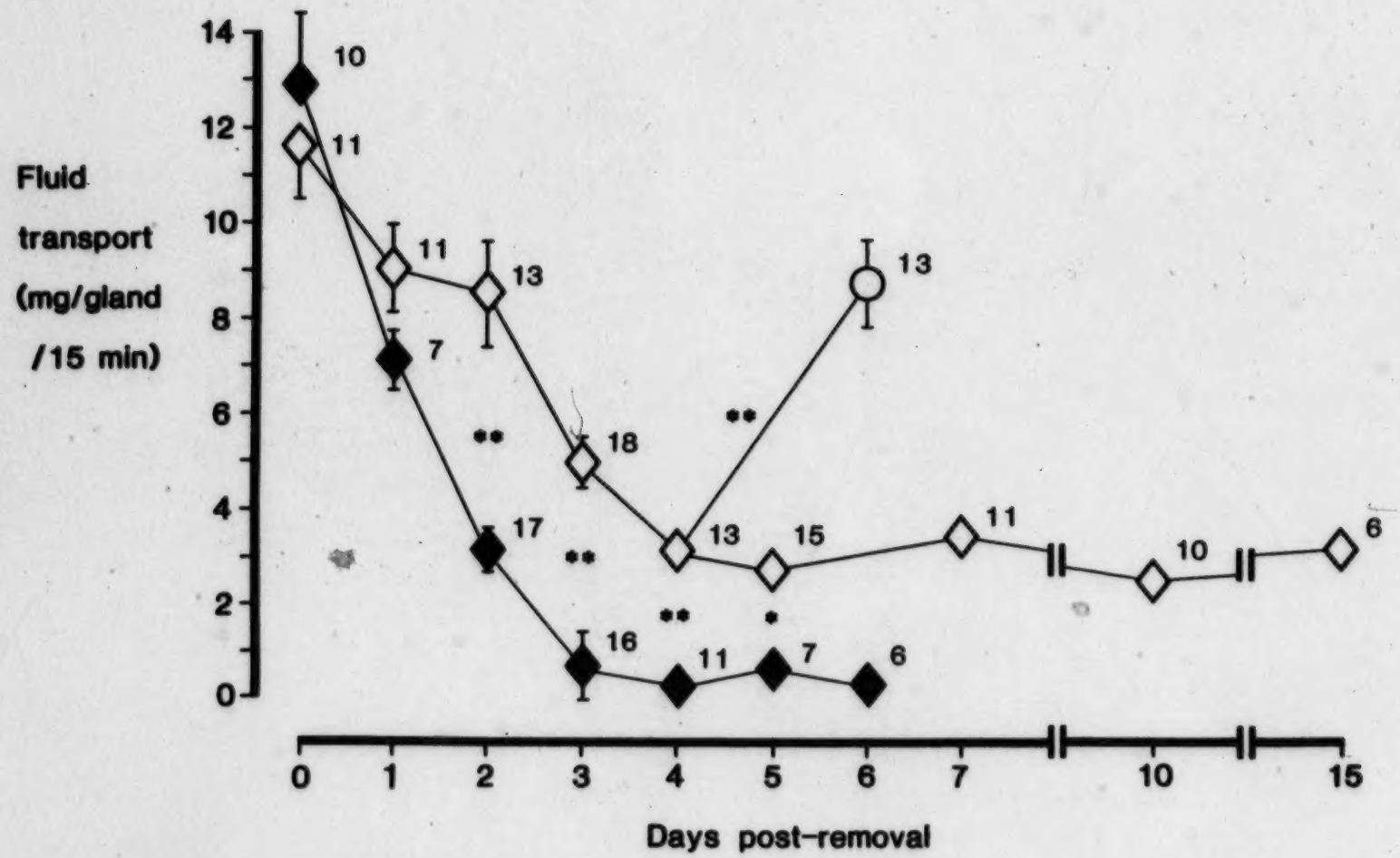

Fluid transport (mg/gland /15 min)
14
12
10
8
6
4
2
0
10
11
11
13
7
18
13
13
15
11
10
6
17
16
11
7
6
**
**
**
**
*
0
1
2
3
4
5
6
7
10
15
Days post-removal

Fig. 4. Secretory competence of salivary glands of small partially fed ticks implanted into the haemocoel of both small partially fed ticks and replete ticks. Gland implanted into replete ticks transported less than glands implanted into small partially fed ticks. Mean, SE and N are given for each. Bars under a solid line are not significantly different ($p>0.05$). (*) $p<0.05$. Salivary glands were tested 2 days after they were implanted.

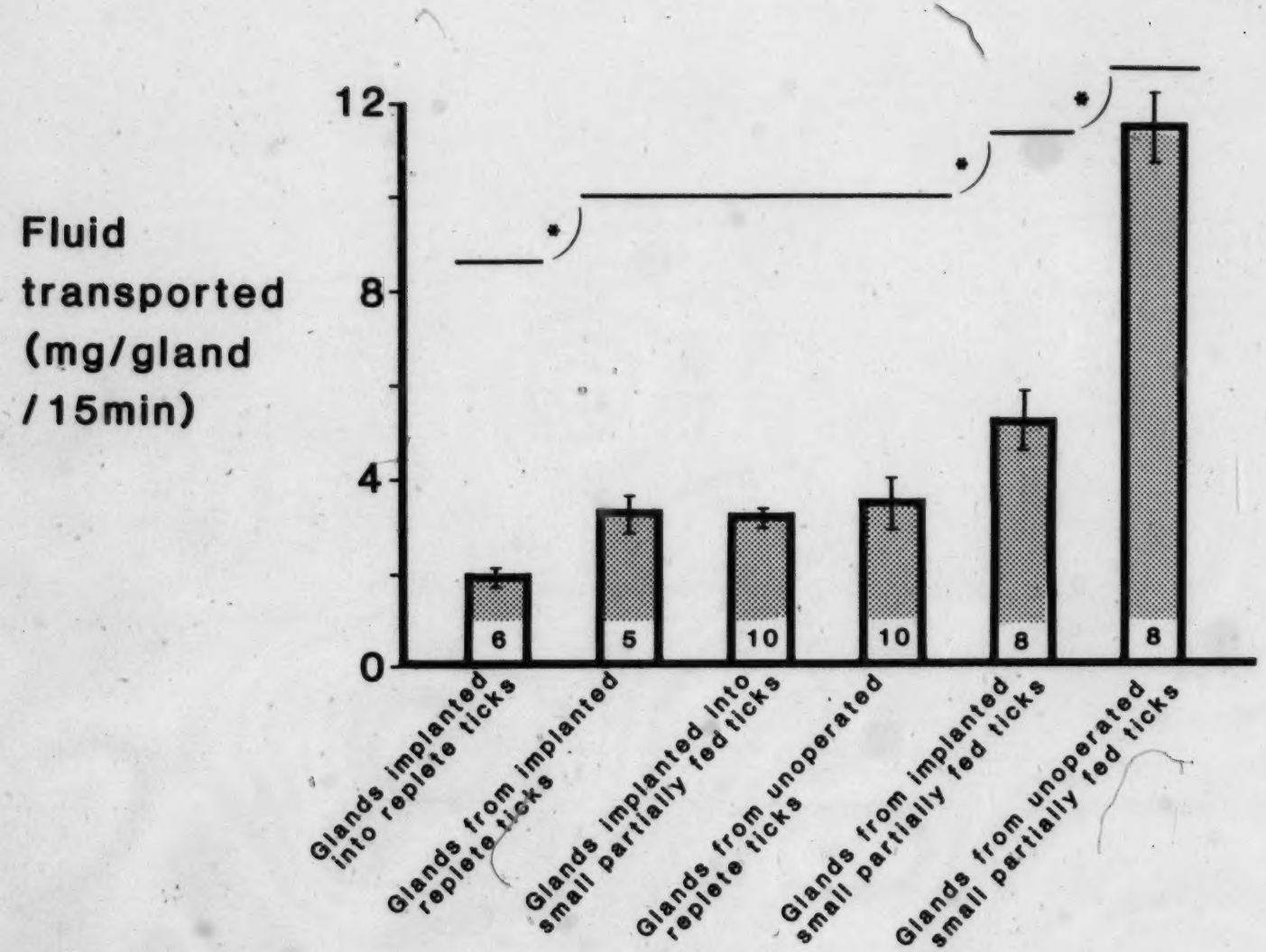

Fluid transported (mg/gland /15min)
12
8
4
0
*
*
*
6
5
10
10
8
8
Glands implanted into replete ticks
Glands from implanted replete ticks
Glands implanted into small partially fed ticks
Glands from unoperated replete ticks
Glands from implanted small partially fed ticks
Glands from unoperated small partially fed ticks

Fig. 5. Secretory competence of small partially fed ticks joined in parabiosis to either replete ticks or other small partially fed ticks. Small partially fed ticks joined to replete ticks transported less than small partially fed ticks joined to other small partially fed ticks. Mean, SE and N are given for each. Statistics as in figure 4. Salivary glands were tested 3 days after parabiosis.

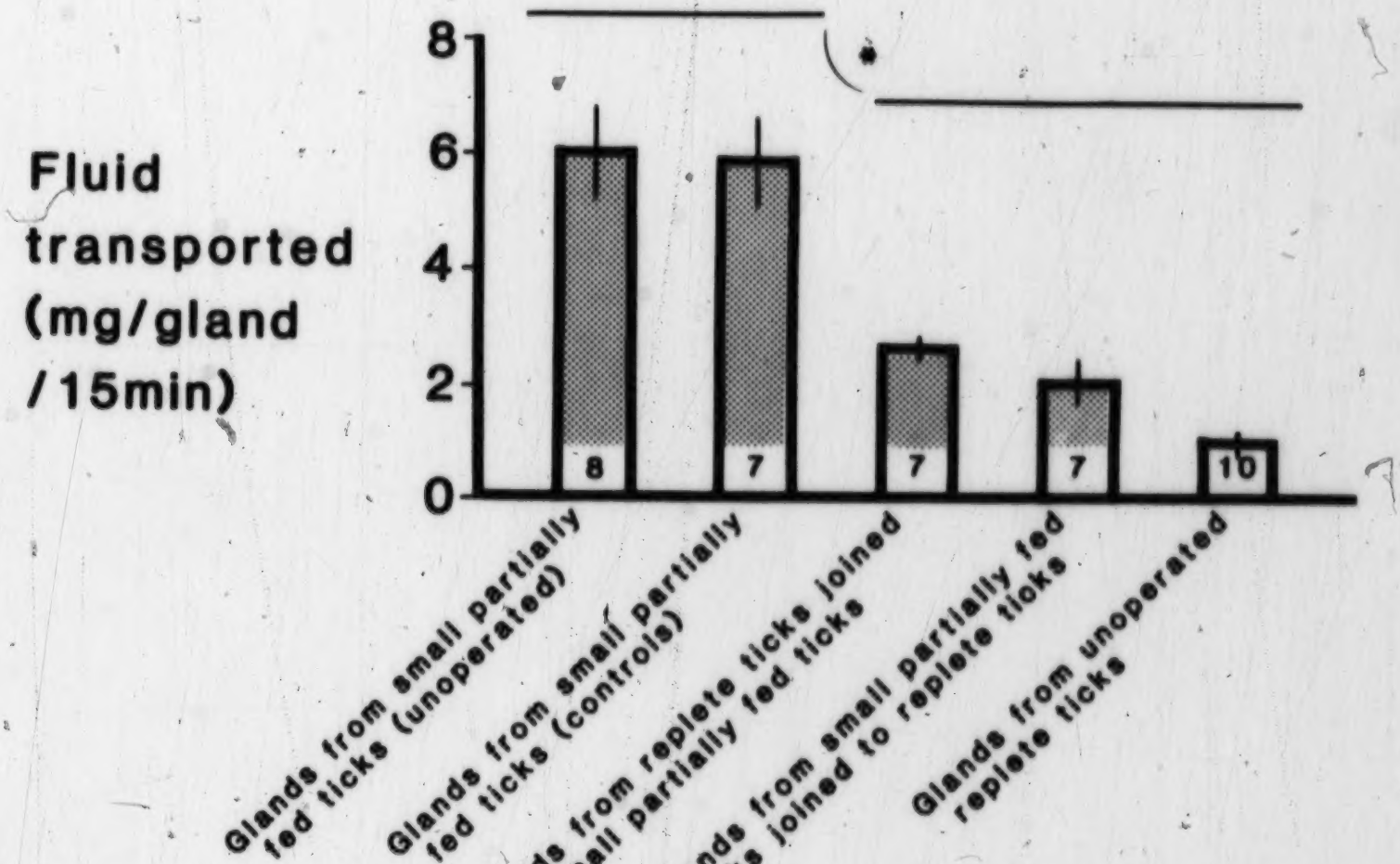
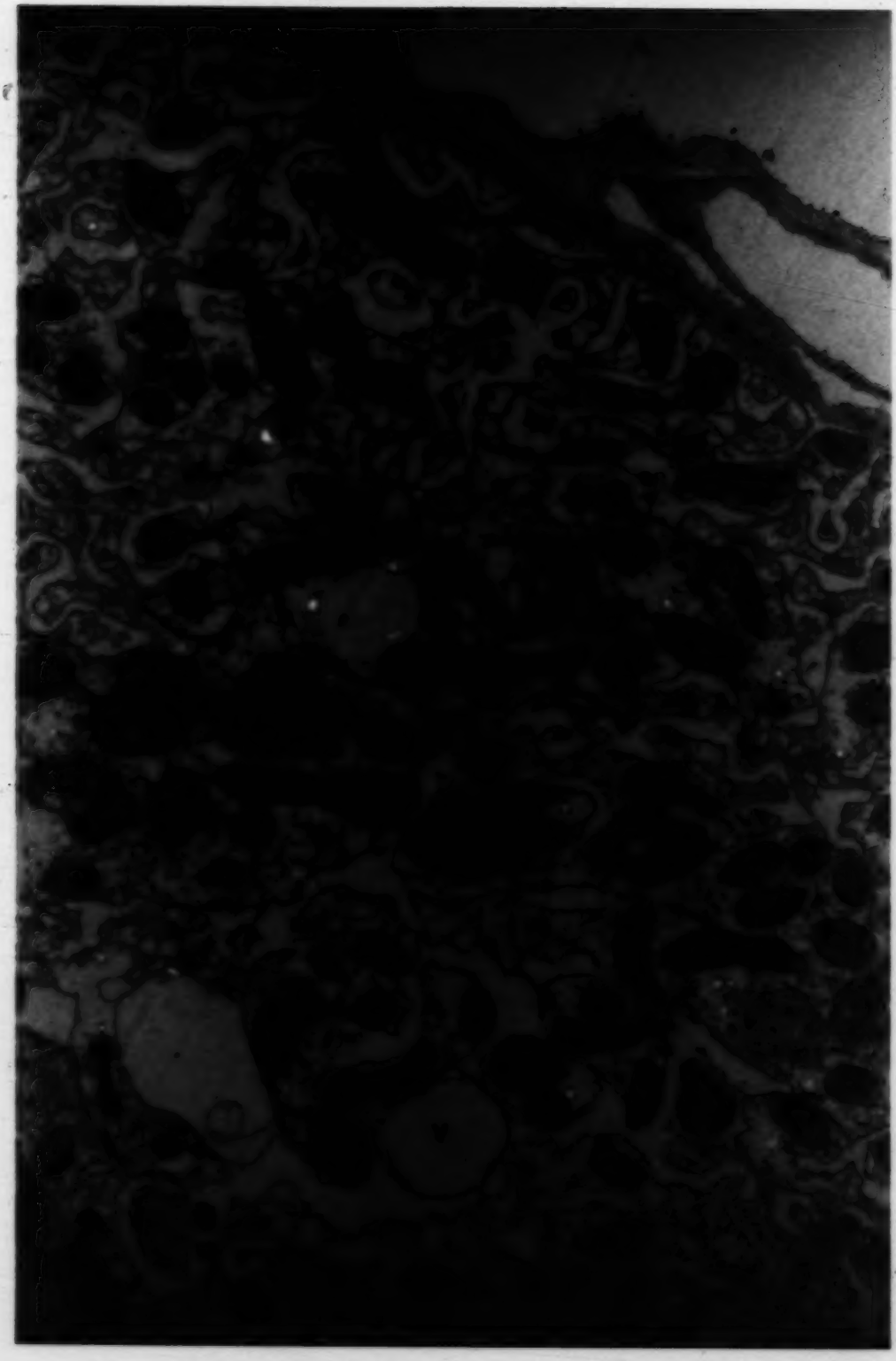

Fluid transported (mg/gland /15min)
8
6
4
2
0
8
7
7
7
10
*
Glands from small partially fed ticks (unoperated)
Glands from small partially fed ticks (controls)
Glands from replete ticks joined to small partially fed ticks
Glands from small partially fed ticks joined to replete ticks
Glands from unoperated replete ticks

Fig. 6. Appearance of the secretory labyrinth in the type III acinus of a small *Amblyomma hebraeum* removed from the host for 5 days. Note the lack of autophagic activity (see fig. 13 for comparison). Abbreviations as follows: *L*, primary lysosome; *M*, mitochondrion; *V*, vacuole (of unknown function); *A*, autophagic vacuole. (Mag. X 19,800)



III. Chapter Three

Neural Involvement in the Control of Salivary Gland Degeneration

A. Introduction

As mentioned in the previous chapter, salivary gland degeneration did not occur for at least 15 days in small partially fed ticks (fig. 3), yet was virtually complete in engorged ticks 5 days after they had detached from the host. This suggests that the quantity of blood imbibed plays a decisive role in determining the course of salivary gland degeneration. In many other blood-sucking arthropods, the size of the blood-meal triggers physiological events. For example, in replete *Rhodnius*, stretch in the abdominal musculature stimulates mechanoreceptors which signal a neurosecretory center in the CNS, resulting in the release of a diuretic hormone (Maddrell, 1963). If an analogous system controlling the release of TSGDF were present in *Amblyomma hebraeum*, then cutting the nerves which innervate the abdominal musculature should block salivary gland degeneration. This chapter describes experiments designed to

test this hypothesis.

B. Materials and Methods

1) Procedure for Staining Neural Tissue

Ticks were dissected so as to expose the synganglion (the synganglion is composed of all the characteristic arthropod ganglia fused into one mass surrounding the esophagus). The tick was then flooded with an aqueous solution of 0.001% Janus Green B, for approximately 2 minutes. This procedure selectively stains neural tissue. Nerves paths were then followed under the dissecting microscope.

2) Surgical Procedure for Nerve Disruption

Ticks were suspended in the hypobaric chamber (fig.1) with the area between the mouthparts and the genital pore exposed. A semi-circular incision, as indicated in figure 1, was made with a fine, razorblade scalpel. After folding back the cuticular flap, gentle retraction of some anterio-ventral lobes of gut exposed the synganglion to view.

Groups of nerves were located and severed at their juncture with the synganglion. Following surgery the

incision was sealed with glue. Glands of operated ticks were assayed for secretory competence 4 days after surgery, and the results compared to those of sham-operated ticks. In each case, the synganglion was inspected to confirm the surgical treatment.

C. Results

Individual *Amblyomma hebraeum* usually terminate feeding at night (Rechav, 1978). This could result in a delay of up to 18 hours between detachment and surgery. Data which will be discussed later (chapter 5) indicated that a delay of between 12 and 24 hours was sufficient to cause almost total salivary gland degeneration. Since salivary glands of small partially fed ticks did not degenerate for at least 15 days (fig. 2) and salivary glands of engorged ticks did, I next tested salivary glands from partially fed ticks of intermediate weights. It was my hope to find a population of partially fed ticks which would undergo complete salivary gland degeneration within 4 days. In this way, surgery could be performed with little delay following removal from the host.

Glands from females between 0.30-0.40g transported 0.97±0.27mg fluid/gland/15 minutes (n=15). This figure is significantly less than that obtained with salivary glands from small partially fed females (3.10±0.26 mg/gland/15 minutes, n=11; $p<0.05$). However, it is also significantly higher ($p<0.05$) than the results obtained from salivary glands from engorged ticks (0.30±0.10; n=15). Glands from partially fed ticks 0.40-0.90g (hereafter referred to as large partially fed ticks) transported 0.44±0.15mg fluid/gland/15 minutes (n=13), a value which is not

significantly different from that obtained from salivary glands of fully engorged ticks. Thus, all subsequent experiments utilized large partially fed ticks rather than engorged ticks, because I then knew exactly when they had been removed from the host.

The literature does not provide a reasonable concensus on the elements of tick neuroanatomy (Obenchain, 1974; Obenchain & Oliver, 1976; Binnington, 1981). This necessitated that I determine the general pattern of the nerves emanating from the synganglion in *Amblyomma hebraeum*. The structure given in figure 7 is a compilation of diagrams obtained from at least 7 ticks. Individual nerves could not be visualized when using the operative procedure for the physiological experiments; therefore, for these experiments I severed groups of nerves (table 1). All experimental procedures were confirmed by subsequent dissection.

Severing all the opisthosomal nerves (Groups C & D) resulted in significant inhibition of salivary gland degeneration ($p<0.05$). In contrast, severing all the pedal and haemal nerves or the cephalic nerves did not result in significant inhibition of salivary gland degeneration (fig. 8). In several ticks, the surgery inadvertently resulted in a bilateral disruption of the medial opisthosomal nerves (Group D), but only a unilateral disruption of the lateral opisthosomal nerves (Group C). This treatment did not inhibit salivary gland degeneration (0.52 ± 0.20mg fluid/gland/15 minutes; $n=8$, $p>0.05$) as compared to sham

operated controls of the same weight range (0.44±0.15mg fluid/gland/15 minutes; n=12). I next tested whether salivary gland degeneration was blocked by disruption of the lateral opisthosomal nerves only. Thus, I bilaterally severed either the lateral opisthosomal nerves, or the medial opisthosomal nerves (Group C or D respectively). Severing either group alone did not inhibit salivary gland degeneration (fig. 8).

D. Discussion

A neural component in the pathway controlling salivary gland degeneration appears to exist in the opisthosomal nerves. Possibly stretch receptors in the periphery signal the synganglion that feeding has progressed past a critical level. A similar system controlling the release of diuretic hormone has been described in *Rhodnius prolixus* (Maddrell, 1963). An alternative hypothesis would be that there are multiple release sites of TSGDF scattered in the opisthosoma. Again, an analogous system can be found in *Rhodnius*; abdominal neurosecretory organs, found in the second through fifth segments, release an antigonadotropin (Davey & Kuster, 1981; Davey, 1981). At the present time I favour the first alternative because all the opisthosomal nerves, with the possible exception of the nerves supplying the genital tract, appear to terminate in somatic muscle bundles. Unless these muscles also function as neurohaemal organs, it is more attractive to suggest that they do indeed harbor stretch receptors. One population of stretch receptors which triggers salivation when fluid is imbibed has been proposed to exist in the idiosoma of *Amblyomma hebraeum*. Faced with a large injected volume of isosmotic saline (25µl/100mg body weight), ticks will secrete saliva (Kaufman *et al*, 1980). This response can be almost totally abolished by severing the opisthosomal nerves (Kaufman &

Harris, Can. J. Zool., 1983, in press).

Fig. 7. Structure of the synganglion of *Amblyomma hebraeum*. Major nerve trunks were traced under the dissecting microscope. Terminology follows that of Binnington (1981). Nerves are identified as follows: Pedal nerve (PN), haemal nerve (HN), salivary gland nerve (SGN), lateral segmental organ (LSO). The opisthosomal nerves have been divided into two groups: the lateral opisthosomal nerves comprise a nerve to the genital pore (GP), a nerve to the seminal receptacle (SR), and the paraspiracular nerve (PS). The medial opisthosomal nerves innervate the lateral dorso-ventral muscles (LDVM) and the medial dorso-ventral muscles (MDVM). The cephalic nerves serve the palps, eyes, chelicerae, salivary glands and Gene's organ (GO). The synganglion is surrounded by the neurolemma (NL). Tracheae (T) and the esophagus (E) are also indicated.

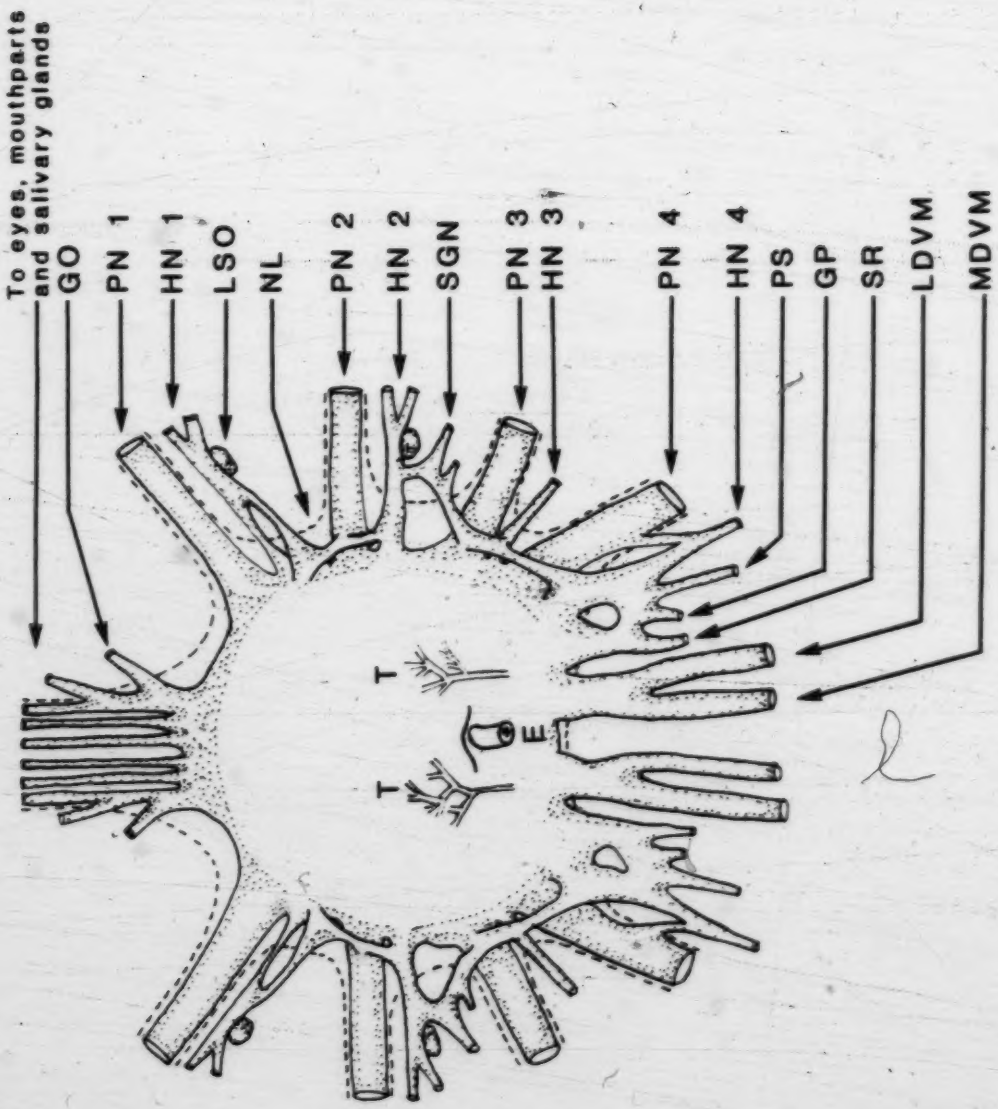
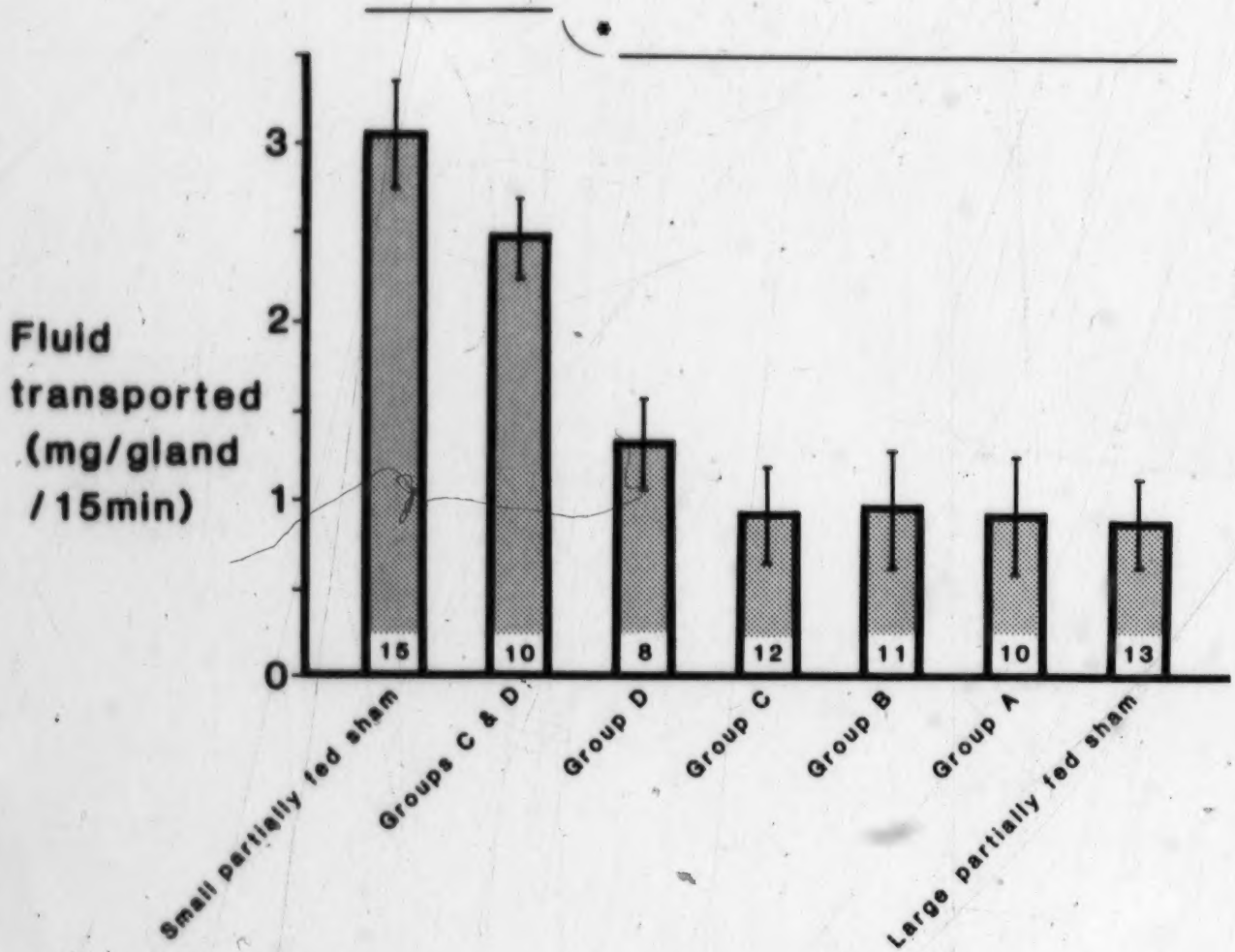

To eyes, mouthparts and salivary glands
GO
PN 1
HN 1
LSO
NL
PN 2
HN 2
SGN
PN 3
HN 3
PN 4
HN 4
PS
GP
SR
LDVM
MDVM
T
T
E

Table 1

Groups of nerves which were severed in the nerve disruption experiment

Groups	Nerves	Organs Innervated
A	cephalic	palps eyes chelicerae Gene's organ integument
B	pedal + haemal	legs coxal muscles salivary glands integument
C	lateral- opisthosomal	seminal receptacle genital pore spiracle integument
D	medial- opisthosomal	dorso-ventral muscles (medial & lateral)

Fig. 8. Fluid secretory ability of salivary glands extirpated 4 days after various groups of nerves were severed. For a definition of experimental groups see table 1. It is only by cutting all the opisthosomal nerves (groups C & D), that salivary gland degeneration could be attenuated. Mean, SE and N are given. Statistics as in fig. 4.


Fluid transported (mg/gland /15min)
3
2
1
0
*
15
10
8
12
11
10
13
Small partially fed sham
Groups C & D
Group D
Group C
Group B
Group A
Large partially fed sham

IV. Chapter Four

Involvement of Mating in the Control of Salivary Gland Degeneration

A. Introduction

In virtually all ixodid ticks, mating takes place on the host after the blood-meal has commenced. In the only genus in which mating need not take place on the host (*Ixodes*), mating must take place before normal feeding can proceed (Balashov, 1972). As already mentioned, the blood-meal normally consists of two phases. During the initial slow phase which lasts 5-6 days, the female slowly increases in weight to approximately 10% of its final engorged weight (Balashov, 1972); however, if copulation does not occur, the rapid phase of engorgement is not entered. Blood intake may simply be suspended, as in *Dermacentor variabilis* (Pappas & Oliver; 1971, 1972), or slow feeding may persist until the animal reaches a body weight close to normal repletion, as in *Hyalomma excavatum* or *H. dromedarii* (Rechav, 1968; Aboul-Nasr & Bassal, 1972). Even in the facultative parthenogenic species, such as *H. longicornis*, rapid engorgement is not attained unless mating

takes place (Oliver, 1971).

Since development and degeneration of the salivary gland is so closely aligned with the feeding-reproductive cycle, I hypothesized that mating may influence salivary gland resorption. Data obtained by Kaufman (1982) supports this hypothesis; unmated females which had fed to at least 0.40g do not lose secretory competence beyond the level characteristic of small partially fed ticks. Mated ticks of this size, as we have just seen in the previous chapter, do suffer salivary gland resorption.

As was demonstrated in chapter two, any potential signal associated with mating and originating with the seminal receptacle is probably not neural in origin, since severing the nerves to the seminal receptacle (Group C) did not result in inhibition of salivary gland autolysis. Pappas and Oliver (1972) showed that rapid engorgement in ticks is triggered by the transfer of the spermatophore and not by simple tactile stimulation. I therefore thought it unlikely that any stimulus triggering salivary gland degeneration would be associated merely with the tactile sensations of mating. Thus, experiments described in this chapter address the hypothesis that the signal for salivary gland degeneration associated with mating is chemical and is transferred from the male to the female during copulation.

B. Materials and Methods

1) Surgery

Large partially fed ticks were restrained in the hypobaric apparatus (fig. 1), with the area between the mouthparts and the genital pore exposed as the operative site. A semi-circular incision was made around the genital pore using a fine razorblade scalpel. The seminal receptacle was visible upon retraction of the flap of cuticle. The vagina was ligated with fine silk thread to prevent leakage of the contents of the seminal receptacle, and the vagina was severed distal to the ligature. The seminal receptacle was removed, after which the incision was sealed with glue. Sham operations were performed on similar sized females. In some individuals, the seminal receptacles were replaced in the haemocoel following complete extirpation.

2) Injection of Male Tissue Extracts

Fed males were immobilized in petri dishes as previously described (chapter 2). After being flooded with 1.2% NaCl, the dorsum was removed with a razorblade scalpel and discarded. Salivary glands and genital tracts were dissected out and stored in ice-cold 1.2% saline until homogenization. Organs were homogenized in ice-cold 1.2% NaCl, one

organ/20µl. Homogenates were then centrifuged at 12,000g for 5 minutes. The supernatant was recovered, and diluted to one organ/25µl. A 250µl Hamilton syringe was fitted with a 30 gauge hypodermic needle, and filled with the desired extract. The needle was inserted into the haemocoel through the articulation between the capitulum and the scutum. Each female was injected with 25µl of tissue extract. The needle was withdrawn 2 minutes later so as to minimize the loss of fluid from the insertion wound.

C. Results

The seminal receptacles of large partially fed females were extirpated immediately after the ticks were removed from the host. In all cases, the removed seminal receptacles were examined for the presence of a spermatophore, thus insuring that only mated ticks were used. Salivary glands from such females (which were tested 4 days later) transported 2.7±0.5 mg/gland/15 min. (n=14). This figure is not significantly different ($p>0.05$) from the rate of transport found in small partially fed females (fig. 9). Salivary gland degeneration in large partially fed ticks can be totally restored simply by replacing the extirpated seminal receptacle (fig. 9).

In order to determine if the chemical signal suggested by the previous experiment is introduced from the male, I removed the seminal receptacles from large partially fed ticks immediately upon removal from the host. I then immediately injected these ticks with an extract of male genital tracts. Control ticks were prepared in the same manner, with the exception that they were injected with an extract of male salivary glands. Fluid secretion of salivary glands from the females injected with male salivary gland homogenates was not significantly different ($p>0.05$) from the value observed for glands from small partially fed ticks (fig. 9) Those females injected with homogenates of male

genital tracts, however, transported significantly less than the controls injected with male salivary gland extract ($p<0.05$; fig. 9).

D. Discussion

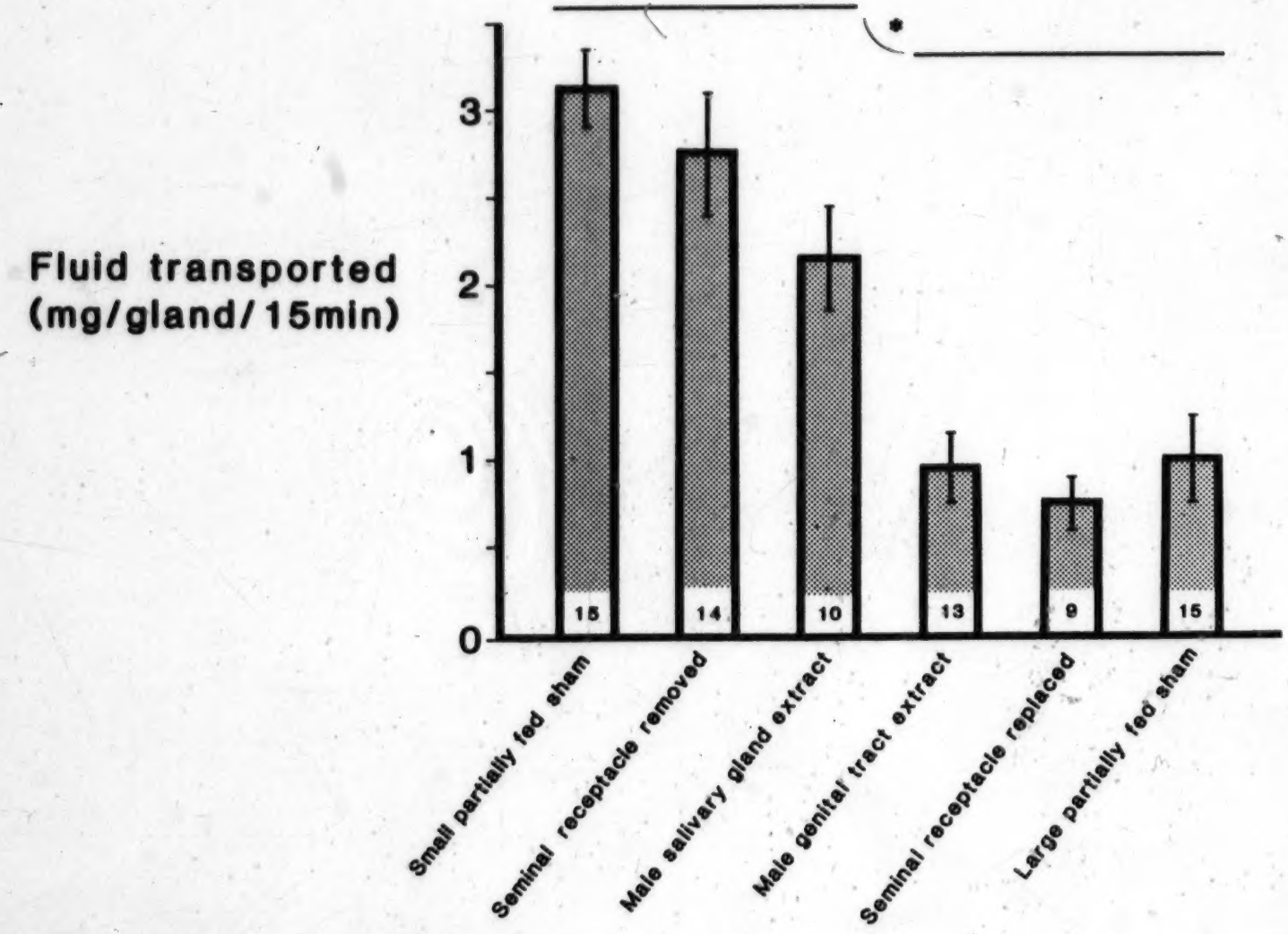
In many insects, chemical factors which signal that mating has occurred are produced in the genital tract of the male and transferred to the female (see review by Gillott & Freidel, 1977). For example, in *Melanoplus sanguinipes*, a substance produced in the male accessory gland, transferred to the female during copulation causes a stimulation of oviposition. (Friedel & Gillott, 1976) In *Rhodnius*, however, the bursa copulatrix releases a hormone when a spermatophore is deposited in it (Davey, 1965). The former appears to be the case with *Amblyomma hebraeum* since salivary gland degeneration can be induced in large partially fed females which have had their seminal receptacles removed, simply by injecting an extract of fed male genital tracts. This response does not appear to be a non-specific reaction to extracts of male tissues, since the extracts of male salivary glands did not result in salivary gland degeneration.

These data suggest that a chemical factor, hereafter referred to as 'mating factor' (MF), is produced in the genital tract of the male, and is transferred to the female via the spermatophore. MF is required before salivary gland degeneration can proceed.

The seminal receptacle remained intact in those females in which salivary gland degeneration was blocked by

disruption of the opisthosomal nerves. Innervation is not required for the release of MF, since extirpation, which results in the severing of all the nervous connections, and subsequent replacement of the seminal receptacle allowed salivary gland degeneration to proceed. In those ticks in which salivary gland degeneration was blocked by the disruption of their opisthosomal nerves, the seminal receptacle was still intact. One can assume that in these ticks, MF was still present to exert its influence and yet salivary gland degeneration did not occur. This suggests that MF and TSGDF are 2 distinct factors.

Fig. 9. Influence of the seminal receptacle on salivary gland degeneration. Seminal receptacles were extirpated from mated, large partially fed ticks. In some cases, the excised seminal receptacles were replaced in the haemocoel. In the latter ticks, the salivary glands lost secretory competence. In other cases, the females were injected with extracts of male genital tracts or male salivary glands. Only the injection of extracts of male genital tracts resulted in salivary gland degeneration. Mean, SE and N are given, Statistics as in fig. 4.


Fluid transported
(mg/gland/15min)
3
2
1
0
*
15
14
10
13
9
15
Small partially fed sham
Seminal receptacle removed
Male salivary gland extract
Male genital tract extract
Seminal receptacle replaced
Large partially fed sham

V. Chapter Five

A Possible Candidate for Tick Salivary Gland Degeneration Factor

A. Introduction

Until recently, experimental organ culture of tick salivary glands has been primarily utilized for the study of the development of various pathogens which are transmitted during feeding. Kaufman and Barnett (1977) were the first to describe an organ culture technique for tick salivary glands. They assessed the health of each salivary gland after culture by monitoring the rate of salivary fluid secretion. They showed that glands remained viable for up to 2 weeks in culture using modified TCM 199. Bell (1980) described a "backless tick explant" method for culturing tick tissue. In essence, the dorsal cuticle is removed and the rest of the tick is submerged in culture medium. Thus, the nervous system, gut diverticula, reproductive tract and salivary glands remain intact, yet one can replace the haemolymph with artificial media. Ultimately, I adopted this method to test the effects of potential stimuli on salivary gland degeneration. In particular I was able to test (at least partially) whether stretch in the gut epithelium might

be the signal associated with salivary gland degeneration, or whether salivary gland degeneration might be mediated by digestion products from the gut in the fluid bathing the salivary glands.

As mentioned in the previous chapter, TSGDF is distinct from MF, and is probably released as a result of both stretch of the idiosoma and exposure to MF. Preliminary data obtained by Dr. P. A. Diehl (University of Neuchatel) suggest that ecdysteroid titers begin to rise as female *Amblyomma hebraeum* enter the rapid phase of engorgement (at approximately 0.35g body weight). This corresponds well with the approximate weight above which salivary gland degeneration will be initiated. Thus ecdysteroids appeared to be potential candidates for TSGDF. Using organ culture I tested the effects of ecdysone and 20-hydroxyecdysone on salivary gland function.

B. Materials and Methods

1) Organ culture

Salivary glands were cultured according to the "backless tick explant" method of Bell (1980). Ticks were removed from the host, washed in distilled water and surface sterilized in 1% Thimerosal (Sigma) for one minute, followed by 70% ethanol for one minute. The ticks were then transferred to a horizontal laminar flow sterile air cabinet, where they were glued to the bottom of sterile, disposable petri dishes (60 x 15mm; Falcon). The ticks were flooded with sterile TCM 199, and the dorsal cuticle removed with a razorblade scalpel and discarded. Unless stated otherwise, the gut diverticula were also removed and discarded. The ticks were then rinsed with 3 changes of sterile medium, submerged in medium, covered and transferred to an incubator maintained at 26°C and 95% relative humidity. In ticks which were cultured with their gut diverticula intact, the body cavity was filled with 200µl of medium after the sterile rinses. After the culture period, the salivary glands were harvested, and assayed for secretory competence. The culture medium was composed of TCM 199 (Gibco), containing 2.1g NaCl/l, and buffered with 10mM morpholinopropanesulphonic acid (MOPS; Sigma). The medium also contained penicillin (5000 units/l) and streptomycin

(5000µg/l). Ecdysone or 20 hydroxyecdysone was first dissolved in 70% ethanol to form a stock solution (1000µg/ml). Different volumes of the stock solutions were added to the culture medium to give the desired ecdysteroid composition. The highest concentration of ethanol that cultured tissues were subjected to was 0.07%. Controls were cultured with 0.07% ethanol in TCM 199.

C. Results

In initial experiments utilizing the backless tick explant technique, I tested how well salivary glands retained secretory function while being cultured in TCM 199 alone. As demonstrated in figure 3, salivary glands from small partially fed ticks lose approximately 40% of secretory function 2 days after removal from the host, and 70% after 4 days off the host. Salivary glands from small partially fed ticks kept in organ culture show virtually the same profile for loss of function as unoperated ticks (table 2).

In order to determine if stretch in the gut epithelium or the presence of digestion products are critical stimuli affecting salivary gland degeneration, large partially fed ticks were cultured with their gut diverticula intact. In this series of experiments, the haemocoel was filled with 200µl of culture medium. This volume is larger than the haemolymph volume found in large partially fed ticks, but is similar to the haemolymph volume found in engorged ticks (approximately 10% of the body weight; Kaufman et al, 1980). Fluid transport by salivary glands of such ticks cultured for 4 days (table 2) was virtually identical to the value determined for small partially fed ticks 4 days after removal from the host (see fig 3). However, this level of salivary gland function is significantly higher ($p<0.05$) than the level of secretory function seen in large

unoperated partially fed ticks left off the host for 4 days (table 2).

As already mentioned in chapter 3, the degree of engorgement of the female has a profound effect on salivary gland degeneration. Ticks above 0.40g underwent salivary gland degeneration as rapidly as engorged ticks. Thus, I tested whether large partially fed ticks would undergo salivary gland degeneration if cultured at various times after removal from the host. This treatment tested not only whether salivary glands of large ticks would undergo degeneration in culture, but also whether a critical period was required, after the ticks were removed from the host, to initiate salivary gland degeneration, and allow it to proceed in the subsequent absence of TSGDF. These ticks were cultured in 5 ml of medium. All ticks were stored at 26°C and at 95% relative humidity between the time they were removed from the host, and the initiation of organ culture.

As can be seen in figure 10, placing large partially fed ticks into culture within 12 hours of removal from the host completely inhibited salivary gland degeneration. However, a 24 hour delay before the initiation of organ culture was sufficient to allow virtually complete salivary gland degeneration ($p<0.01$).

Figure 11 illustrates the effect of a mixture of ecdysteroids on secretory function of salivary glands from small partially fed ticks kept in organ culture. A mixture of both ecdysone and 20-hydroxyecdysone (1:1, wt/wt) was

used since both are present in adult ticks (P.A. Diehl, personal communication). A dose dependent relationship exists between the loss of salivary function and the concentration of ecdysteroids. Ticks cultured with ecdysone alone (100 ng/ml) transported 1.40±0.50 mg/gland/15 min. (n=6) whereas ticks cultured with 20-hydroxyecdysone alone transported 1.52±0.51 mg/gland/15 min. Neither of these values is significantly different ($p>0.05$) from the rate observed in ticks cultured with a mixture of both forms of ecdysone (100 ng/ml total ecdysteroid content, fig. 11).

D. Discussion

Salivary glands kept in organ culture for up to 4 days using the backless tick explant method secrete just as well as those from unoperated ticks kept off the host for the same period of time. Thus, this method proved excellent for testing factors which induce salivary gland degeneration. Interestingly, organ culture does inhibit salivary gland degeneration in large partially fed ticks, regardless of whether the gut diverticula are intact or removed. The nerves which innervate the gut wall arise from the synganglion adjacent to the exit of the esophagus. These nerves were left intact in animals which did not have their gut diverticula removed; therefore, any putative neural signals resulting from stretch in the gut diverticula would presumably still have been transmitted to the synganglion. In these ticks salivary gland degeneration did not occur, suggesting that stretch in the gut wall does not trigger the release of TSGDF. Also, gut activity appeared to be unaffected since spontaneous muscular activity was observed.

Presumably, digestion products were being released into the medium, yet salivary gland degeneration did not occur. The latter suggests that such products do not trigger salivary gland degeneration. Moreover, salivary gland degeneration probably cannot be attributed to a lack of MF since the seminal receptacles of all the cultured ticks were

intact, yet salivary gland degeneration was indeed inhibited in large partially fed ticks in culture. In addition, the system which suffers the most disruption with this culture method is the dorso-ventral musculature of the idiosoma, where virtually all the muscle bundles are severed. If, as proposed in chapter 3, stretch receptors are present in these muscles it is reasonable to assume that destruction of the muscle bundles would eliminate the signals from the stretch receptors, resulting in an inhibition of salivary gland degeneration.

Salivary gland degeneration is not inhibited by the culture medium *per se*, as it will proceed in large partially fed ticks put into culture more than 12 hours after removal from the host. Thus, it would appear that either a critical period of exposure to TSGDF is required or a critical latent period exists before TSGDF is released. However, once degeneration has been triggered, it proceeds to completion. In this experiment, any glands which lost secretory function, possessed levels of transporting ability which were consistant with total salivary gland degeneration (refer to table 2, large partially fed ticks off the host for 4 days). If salivary gland degeneration were simply halted at the moment that organ culture was begun, such glands would maintain the level of secretory function which they presumably possessed when put into culture (refer to days 1 and 2 for partially fed ticks in fig. 3).

As previously mentioned in this chapter, haemolymph ecdysteroid titers rise from a basal level of approximately 10 ng/ml, during feeding, to 300-500 ng/ml, by 6 days after termination of feeding (P.A. Diehl, personal communication). Similar concentrations of ecdysteroids cause a dramatic loss of function in salivary glands of ticks kept in organ culture (fig. 11). All these ticks were still capable of independent movement of their legs and the general appearance of the tissue was healthy, indicating that the loss of function was not due to a general necrosis of the tissues. Thus, the effect of ecdysteroids appears specific, and so an ecdysteroid is a likely candidate for TSGDF. However, no difference in activity could be detected between ecdysone and 20-hydroxyecdysone in this study. It must be noted that all the tissues of the tick were intact, thus any enzymatic conversion of one form of the hormone to another would also be possible.

Table 2

Effect of organ culture on salivary gland function of partially fed ticks.

Days after removal from host		Fluid transported (mg/gland/15 min.)	
	unoperated ticks		organ culture in TCM 199
2	8.5±1.16 (n=13)	p>0.05	7.2±0.79 (n=10)
4	3.1±0.26 (n=13)	p>0.05	2.88±0.33 (n=18)
	cultured with gut diverticula intact		
4	3.30±0.65 (n=6)		

Fig. 10. Secretory ability of salivary glands from large partially fed ticks put into culture at various times after removal from the host. All salivary glands were tested 96 hours after removal of the tick from the host. Salivary gland degeneration did not occur in ticks put into culture up to 12 hours after removal from the host. However, degeneration proceeded virtually to completion in ticks placed in culture 24 or more hours after removal from the host. Mean, SE and N are given.
(*) Denotes a significant loss of secretory function as compared to salivary glands from ticks put into culture immediately after removal from the host ($p<0.01$).

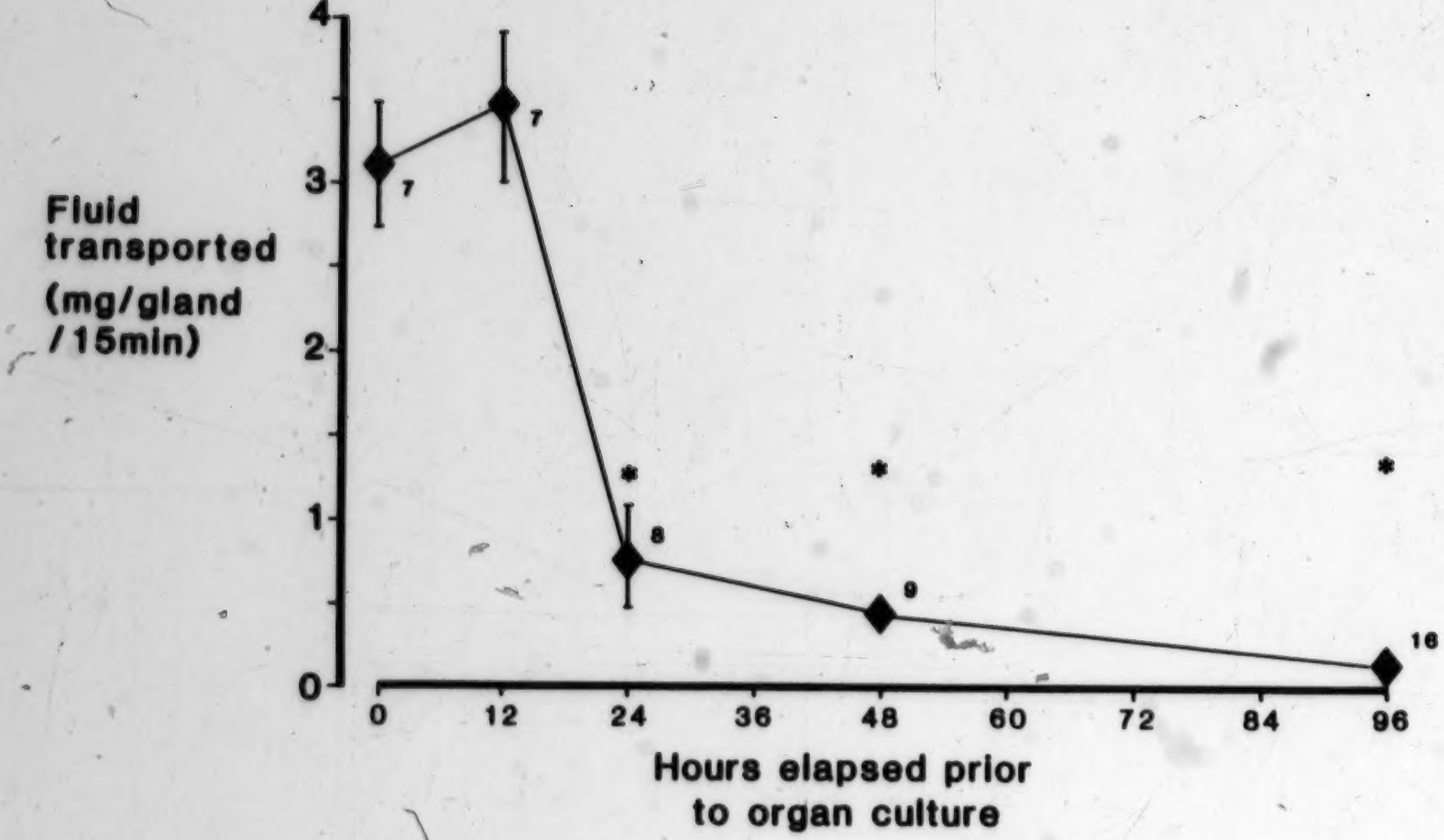
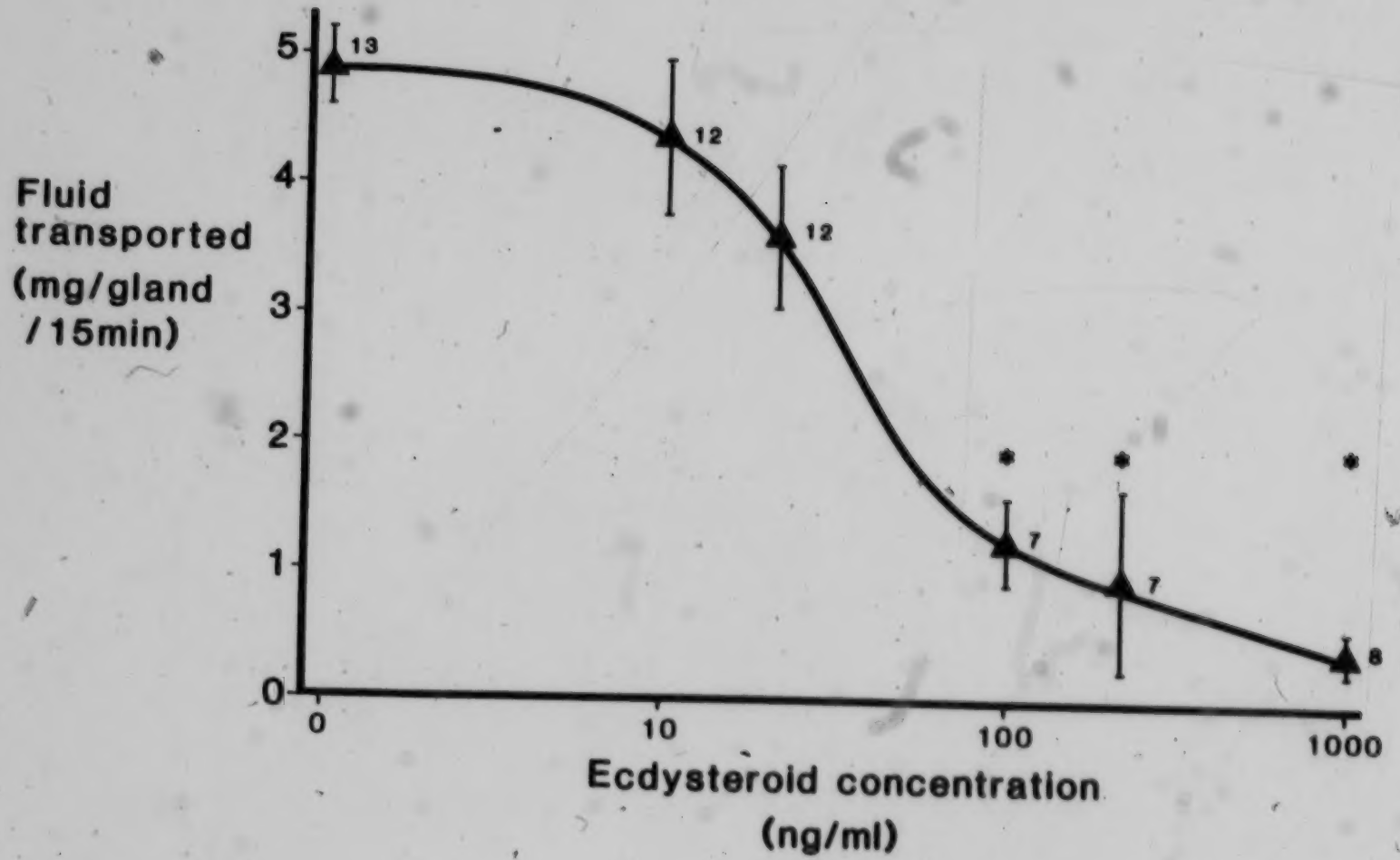

Fluid transported (mg/gland /15min)
4
3
2
1
0
7
7
8
9
16
*
*
*
0
12
24
36
48
60
72
84
96
Hours elapsed prior to organ culture

Fig. 11. Secretory ability of salivary glands from ticks kept in organ culture with ecdysone and 20-hydroxyecdysone (1:1, wt/wt). A dose dependent loss of secretory function was caused by the ecdysteroids. Ecdysteroid concentrations in haemolymph of replete *Amblyomma hebraeum* rises from approximately 8 ng/ml during the slow phase of feeding to approximately 350 ng/ml on day 4 post-repletion (P.A. Diehl, University of Neuchatel, personal communication). Mean, SE and N are given. (*) Denotes a significant loss of secretory function as compared to glands from ticks cultured with 0.07% ethanol ($p<0.01$).


Fluid transported (mg/gland /15min)
5
4
3
2
1
0
13
12
12
7
7
8
0
10
100
1000
Ecdysteroid concentration (ng/ml)

VI. Chapter Six

General Discussion

The assay described in this thesis for monitoring salivary gland degeneration has several advantages over the previous methods: it is quantitative, simple and fast. Previously, salivary gland activity was tested by injecting rather large amounts of dopamine into intact animals and measuring salivation (Kaufman & Harris, Can. J. Zool., in press), or suspending intact salivary glands, including the duct and cuticular orifice, in a droplet of medium surrounded by paraffin oil (Kaufman & Phillips, 1973a; see also fig. 12). Measurements of salivary gland function by the former method are undesirable since one is measuring the response to dopamine of the whole animal, not just the salivary glands. The latter method is suitable for testing effects of multiple doses and combinations of drugs, but requires elaborate and time consuming preparation. It was not very amenable to testing the response of many glands to a single dose of dopamine. Thus, the technique described in this thesis to assay salivary gland secretory ability should prove valuable in future studies in which a simple measure of viability is required.

Salivary gland autolysis is accomplished during a period of intense autophagic activity in the area of the type III acinus which is believed to be responsible for

fluid secretion, the ablumenal interstitial cell and the transformed f-cell (fig. 13). As salivary gland degeneration proceeds, secretory function is lost. However, masking the loss of function due to autolysis, was a loss of secretory function which also occurs in small partially fed ticks. This non-specific loss was not total, and was complete by 4 days post-removal from the host. Thus, any loss of function below the level found in small partially fed ticks 4 days off the host was attributed to autolysis.

Salivary gland degeneration is triggered hormonally, with an ecdysteroid being the most likely candidate. A critical weight exists over which the ticks are committed to salivary gland degeneration. Most likely the ticks monitor stretch in the dorso-ventral muscles of the idiosoma. Moreover, salivary gland degeneration did not appear to be triggered by the presence of digestion products, since ticks cultured with their gut diverticula intact did not undergo a loss of secretory function. Also, small partially fed ticks did not appear to undergo salivary gland degeneration for at least 15 days after removal from the host, and yet digestion products would also be present in the haemolymph of these ticks.

Salivary gland degeneration did not proceed unless mating had taken place. The signal associated with mating appears to be chemical, rather than neural, in nature and is most likely incorporated into the spermatophore since virtually no fluid accompanies the spermatophore during

transfer. In fact, the only fluid which is transferred to the female during copulation is a salivary secretion used as a lubricant for the spermatophore (Feldman-Muhsam et al, 1970; Oliver et al, 1974), and yet the injection of extracts derived from the salivary glands of fed males into large partially fed females which had undergone seminal receptacle extirpation, failed to initiate salivary gland degeneration (fig.9).

It must be mentioned here that in both the experiments involving the disruption of nerves, and the ablation of the seminal receptacle, two constraints applied. First, salivary gland degeneration could only be blocked in partially fed ticks between 0.40 and 0.90g. Presumably, the mechanism initiating the release of TSGDF had already been activated in ticks over 0.90g before surgery, and thus could not be inhibited by these treatments. Second, the experimental treatments on ticks between 0.40 and 0.90g had to be performed within 1.5 hours of removal from the host, regardless of the weight of the tick. Experiments conducted on ticks which had been separated from the host longer than 1.5 hours did not result in blocking salivary gland degeneration (unpublished observations). This phenomenon appears to be independent of the weight of the tick, provided that it was over 0.40g, and therefore independent of the amount of time the tick has spent in rapid engorgement. Also, this phenomenon appears to be related to the removal of the tick from the host. This suggests that

there may be a stimulus associated with detachment that initiates the release of TSGDF in those ticks which are mated and have fed sufficiently.

A. Biological Significance

It is unclear why the salivary glands are resorbed at the termination of feeding. Certainly, the small amounts of protein made available for egg production by salivary gland autolysis are trivial compared to the tremendous protein reserves in the blood-meal. Till (1961) noted that the salivary glands of both larvae and nymphs are resorbed after feeding and prior to moulting. Presumably, this allows for the massive growth of the salivary glands which occurs between the instars. Ecdysteroids have been demonstrated in the larvae and nymphs of *Amblyomma hebraeum*, and appear to be linked with the cuticular changes associated with moulting (Diehl *et al*, 1982). Ecdysone has also been implicated in the induction of autophagic activity in isolated insect fat body (Dean, 1978). Ecdysone may have a similar effect on the salivary glands of moulting larvae and nymphs. If this is the case, then it is reasonable to assume that ecdysteroid mediated resorption in adult females simply reflect an neotenic response that was never lost in the adult.

This study demonstrates that both the presence of MF and stretch in the idiosoma are required before salivary gland degeneration begins. Since *Amblyomma hebraeum*, like most ticks, is not parthenogenic, it is advantageous for it to possess a system which will ensure that feeding has occurred in adequate amounts so that sufficient eggs can be produced, before salivary gland degeneration is initiated. In many cases, extreme tick loads result in the death of the host (Love, 1955; Samuel & Barker, 1979). When such a death occurs, it would undoubtedly result in the stranding of many partially fed ticks, which would be too small to produce a viable egg mass. The system controlling salivary gland degeneration described here, would allow such ticks the time required to quest for a new host. As was mentioned in Chapter 1, feeding by ectoparasites is risky. Thus, as a female approaches its final engorged weight, should it be denied the original host, the reproductive benefit of simply producing what eggs it can would probably surpass the risk involved with re-attachment. This system also ensures that mating has taken place before salivary gland degeneration can proceed. Thus the animal is guaranteed that both mating and feeding have been completed before it irreversibly loses its ability to osmoregulate during feeding.

As mentioned in chapter 4, Mf and TSGDF appear to be two distinct factors. Two mechanisms can explain the relationship between MF and TSGDF. As suggested in chapter 3, feeding results in the distention of the dorso-ventral

muscles of the idiosoma. Receptors stimulated by this stretch could signal a center in the synganglion to release TSGDF. However, this response would not occur unless this center were also stimulated by MF. An alternative explanation would also involve stretch receptors signalling the synganglion with TSGDF being released automatically in this case. However, TSGDF would have no effect on the salivary glands unless MF were present to act synergistically.

The results presented here do not support the possibility that MF primes the sensitivity of the salivary glands to TSGDF. If this were the case, then the long period of exposure of the salivary glands to MF, the time btween mating and the initiation of rapid feeding, should have been sufficient to accomplish this sensitisation and yet salivary gland degeneration did not occur in large partially fed ticks unless MF were present at all times.

B. Suggested Experiments

Several areas of study are suggested by these results. Both TSGDF and MF have yet to be positively identified. The organs which secrete these substances also have not been identified. The system which determines the degree of stretch in the idiosoma also deserves more study; in

particular, experiments to locate and identify the suggested stretch receptor must be conducted. In addition, experiments must be conducted to determine the site of action of MF, be it the synganglion or the salivary gland directly.

Fig. 12. *In vitro* salivary gland preparation developed by Kaufman & Phillips (1973a). The salivary duct is gently pulled out of the droplet of bathing medium (containing dopamine) into the surrounding paraffin oil. The volume of the sphere of saliva which forms at the orifice of the duct can be calculated from the diameter, which is measured with the aid of an optical micrometer in the eye-piece of the dissecting microscope. Diagram courtesy of Dr. W.R. Kaufman, University of Alberta.

side view
top view
0
8
16
24
30

Fig. 13. Ultrastructural appearance of the secretory labyrinth in a salivary gland taken from an engorged *Dermacentor variabilis* showing autophagic vaculoes (as indicated by the arrows) in both the ablumenal interstitial cell (AB) and the f cell (F). Micrograph courtesy of Dr. L.B. Coons, Memphis State University. (Mag. X 9,100)

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VIII. Appendix

Salivary glands were harvested, as already described in chapter 2. After extirpation, the salivary glands were blotted on filter paper for 15 seconds and then transferred to modified TCM 199 containing 1μM dopamine and a small amount of ^{36}chloride and ^{14}C inulin (approximately 20,000 CPM of each). Inulin is a suitable extracellular fluid space marker for tick salivary glands (Kaufman et al, 1980; Sauer *et al*, 1974). Glands were then incubated for 10 minutes, after which they were blotted for 15 seconds, washed for 10 seconds in TCM 199 (without ^{36}Cl and ^{14}C inulin) and then blotted again for 15 seconds. The tissue was then homogenized in 150 μl 1N NaOH. A 25 μl aliquot was removed for protein determination (Bradford, 1976). The remaining homogenate was transferred to 10 ml Brays solution (Bray, 1960) and counted in a Beckman 9000 Liquid Scintillation Counter. The ^{14}C and ^{36}Cl windows were set such that 1.1% of the ^{36}Cl counts were due to ^{14}C, and 6.4% of the ^{14}C counts were due to ^{36}Cl. The original ratio of ^{14}C/^{36}Cl in the incubation medium was determined by counting a 10 μl sample of the incubation medium. Active ^{36}Cl transport was calculated as shown in table 3.

All results are presented as ^{36}Cl CPM/μg protein ± SE. Salivary glands from small partially fed ticks, tested on the day that they were removed from the host, transported 2.3±1.06/10 min. (n=5). This value is not significantly

lower ($p>0.05$) than the value obtained from salivary glands of engorged ticks, 3.4±0.83/10 min. (n=5). Salivary glands tested 2 days after removal from the host showed a dramatic loss of chloride transporting ability. Glands from small partially fed ticks transported 0.6±0.35/10 min. (n=9), whereas glands from engorged ticks transported 0.2±0.18/10 min. (n=9). Again, this difference is not significant ($p>0.05$).

The major problem associated with this assay is that the values obtained for active transport of ^{36}Cl are only about 5% of the total ^{36}Cl uptake. This limits the ability of the assay to detect small differences in rates of active ^{36}Cl transport. This problem may have been compounded by the fact that the main salivary ducts of the glands were not ligated, thus allowing transported ^{36}Cl to return to the bathing medium; this would tend to lower the apparent rates of active ^{36}Cl transport. Another factor complicating the assay, was the fact that the intra-cellular chloride pool proved to be very small compared to the extracellular fluid pool. Thus, a very small error in determining CPM translates to a large error in the active/passive uptake ratio. In short, I considered this assay to be less acurate for the determination of salivary gland secretory competence than the much simpler assay based on net changes in weight described in chapter 2.

Table 3

Calculations for determining active ^{36}Cl transport

Active ^{36}Cl transport was determined with the following equations:

Active ^{36}Cl transport = A - B

Where:

A = ^{36}Cl uptake in the tissue incubated with dopamine

B = ^{36}Cl uptake in the tissue incubated without dopamine

^{36}Cl uptake by the tissue = C - D

Where:

C = total ^{36}Cl in the tissue

D = ^{36}Cl in the extracellular fluid space

^{36}Cl in the extracellular space = (E / F) G

Where:

E = ^{36}Cl in the medium

F = ^{14}C in the medium

G = total ^{14}C in tissue

All counts were corrected for background, as well as crossover of ^{14}C into the ^{36}Cl window and *vice versa*